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CORNELL UNIVERSITY
AGRICULTURAL EXPERIMENT STATION

THE INFLUENCE OF LOW TEMPERATURE ON SOIL BACTERIA

A. F. VASS

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THE INFLUENCE OF LOW TEMPERATURE ON SOIL BACTERIA

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A. F. Vass

Temperature is one of the most important factors influencing the growth and reproduction of microorganisms. Considerable work has been done in studying the effect of low temperature on the vitality and growth of bacteria, but the results have varied so greatly that it is difficult to draw any very definite conclusions from them. Certain investigators have found in frozen soils what they believed to be bacterial growth and reproduction to an even greater extent than is found at temperatures usually considered most favorable for protoplasmic activity.

The increased use of ice and the knowledge of water-borne epidemics have stimulated investigation in regard to the ability of the pathogenic bacteria to withstand freezing and storage in ice. In the work with pure cultures of pathogenic and nonpathogenic bacteria a great variation in results has been obtained. The natural ice dealer can find plenty of evidence to support his theory that the pathogenic bacteria in water are killed when the water is frozen and the ice stored for summer use. On the other hand, the public health officer can present equally strong evidence to show that the bacteria are not killed by freezing.

HISTORICAL

The literature herein reviewed deals with the ability of the pathogenic and the nonpathogenic bacteria to withstand exposure to low temperature, the influence of low temperature on the bacterial flora of the soil, and the cause of the death of the cell upon exposure to low temperature together with the factors that influence it.

PURE CULTURES

In the work with pure cultures both the pathogenic and the nonpathogenic forms were used. In some cases, however, there may be some doubt as to the purity of the cultures.

Regarding the resistance of bacteria to low temperatures, it was found by Schumacher (1874)[1], Göppert (1875), Frisch (cited by Pfeffer, 1903:238), Dewar and McHendrick (cited by Pfeffer, 1903:238), and Meyer (1900), that neither the spores nor the vegetative cells were killed by an exposure of several hours to temperatures ranging from −20° to −200° C.

Pictet and Yung (1884) exposed *Micrococcus lutea* and the vegetative cells of *Bacillus anthracis* to a temperature of −70° C. for one hundred and eight hours; this resulted in the death of the bacteria, as did also a temperature of −130° C. for twenty hours. The spores of *Bacillus subtilis*, *Bacillus anthracis*, and *Bacillus ulna* were not affected by the above-named temperatures. These results verified the conclusion of Melsens (1870), that long exposure of the yeast cells to −91° C. greatly decreases the fermentative activity. The enzyme of the yeast and the toxins of the poisonous bacteria were destroyed by exposure to extremely low temperatures. In his later work with microbes and diatoms, Pictet (1893) found that continued cold at −200° C. did not destroy these.

In studying the bacterial flora of snow, Janowski (1888) noted that continued low temperatures had very little effect on the bacteria. He found several hundred bacteria per cubic centimeter in water from snow.

Forster (1892) noted that the kinds of bacteria able to grow at 0° C. were not very numerous, but they seemed to be widely distributed, especially in water and on the surface and in the intestinal tract of both fresh- and salt-water fish.

In his work with *Bacillus anthracis*, Klepzoff (1895) noted that long exposure to low temperatures reduced the virulence of the organism and that the bacteria in blood and various organs were killed by exposing them to a temperature of −24° C. for twelve days. The colonies on agar plates were destroyed by exposing them for twenty-five days to the above-named temperature.

Kasansky (1899) exposed the pest bacillus and the diphtheria bacillus to temperatures ranging from −10° to −30° C. for five months and found that they were alive at the end of the experiment.

In their investigations with pure cultures of *Bacillus typhosus*, Sedgwick and Winslow (1900) found that from 30 to 60 per cent of the bacteria were destroyed during the first hour of freezing, and at the end of two weeks 99 per cent were destroyed. They studied the effect of alternate

[1] Dates in parenthesis refer to *Literature cited*, pages 1071 to 1074.

freezing and thawing, and noted that it was only slightly more harmful than continued freezing. Park (1901), in working with the same organism, found that all the bacteria were killed when embedded in ice for twenty-two weeks.

Macfadyen (1900) exposed bacteria to the temperature of liquefied air for twenty hours without its affecting their vital properties. The yeast-cell plasma also retained its peculiar properties to effect the production of carbon dioxide and alcohol. In a later report (1903) Macfadyen gives results from using liquid hydrogen, the temperature of this being −252° C. No appreciable effect on the bacteria was noted.

In studying the effect of the storage of ice on the bacteria contained therein, Sparks (1908) concluded that ice, even when cut from water which may contain pathogenic bacteria, is utterly incapable of passing on the disease if it is stored for some time before using. Similar results were obtained by Reudiger, who found that *Bacillus coli* was destroyed by storing in ice for eight weeks, and that the *Bacillus typhosus* organism was dead at the end of thirty-one days.

Budinov (1909) inoculated sterile milk with *Bacterium lactis acidi* and noted the effect of temperature on the life of the organism. At 30° C. most of the bacteria were dead in nine days, and at room temperature very few were alive at the end of eighteen days; while at 0° C. there was no change after thirty days. Alternate thawing and freezing had little or no effect. In such a case, however, temperature acts indirectly. The more favorable the temperature for growth, the greater is the production of acid, which in turn destroys the bacteria.

Poppe (1914) exposed the anthrax bacillus to a temperature of −16° C. for two weeks. The virulence and the reproductive capacity of the organism were not affected.

Jordan (1916) concluded that freezing had about the same effect as slow sand filtration, and that a great majority of the typhoid bacilli perish in a short time in ice, less than 1 per cent remaining alive after three weeks of freezing.

Hilliard, Torossian, and Stone (1915) noted, in their work with *Bacillus coli*, that 99 per cent of the organisms were killed by freezing in tap water for three hours; in the case of *Bacillus subtilis*, 80 per cent were killed under similar conditions. When the bacteria were placed in cream containing 30 per cent of butterfat, a protective action was noted.

Bartram (1916) exposed several of the disease-producing bacteria to winter temperatures for several months. Of the six cultures exposed, only two survived.

SOIL FLORA

Among the early workers who studied the influence of temperature on the bacterial flora of the soil may be mentioned Remy (1902), who studied the seasonal variations. His quantitative study showed no great variation in the different seasons. No samples were taken during the winter.

A correlation between the number of bacteria in the soil and the moisture content was noted by Hiltner and Störmer (1903). Their results indicated also that there was a decrease in the total number of bacteria as the temperature of the soil was lowered. They noted, however, that certain of the frozen samples gave rather high results. Fabricius and Von Feilitzen (1905) found the same correlation to exist between temperature and the bacteria count. Their tests were made during the growing season.

Engberding (1909) concluded, from his studies of bacterial activity in fallow and cropped, and manured and unmanured, plots, that the moisture content was more important than the temperature. Most of his samples were taken during the period of plant growth. Of the two frozen samples studied, the counts were not so high as in some of the unfrozen samples, but they were sufficiently high to show that there are large numbers of bacteria in frozen soil.

Conn (1910) made a careful study of the bacterial flora thruout the year. He concludes from his results that quantitative determinations by means of the gelatin-plate method show that bacteria may be present in the soil in large numbers during the winter, and that there seems to be a rapid multiplication of bacteria in frozen soil. He noted that the number of bacteria increased and decreased nearly parallel with the moisture content of the soil in the frozen samples, but during the winter there was a striking exception to this. In a continuation of the above work (Conn, 1912) his earlier findings were confirmed. The same marked increase in number of bacteria in frozen soil, and decrease in thawed soil, was noted. The increase during the winter was thought to be due to the actual multiplication of the bacteria rather than to a mere rise of the organisms from lower depths brought about by mechanical forces alone.

Conn attempted to divide the soil bacteria into three classes — rapid liquefiers, slow growers, and Actinomyces. He found the greatest increase in the group of slow growers when the soils were frozen. In the quantitative work in the pure cultures he noted that certain types of soil bacteria occur thruout the year, while others apparently exist for short periods only and tend to recur at other times under similar conditions. He suggested the possibility that a different class of bacteria is in the ascendancy in winter from those which are benefited by the warm weather of summer; in which case the increase is not due directly to the low temperature, but to the depressing effect of the cold upon the group of bacteria which is able in summer to keep the winter bacteria in check.

Hutchinson (1911–12) noted that biological changes in the soil take place at a greater rate in the soils of India than in the European soils. The temperature of the India soils ranges from 25° to 30° C. during the growing season, whereas that of the European soils ranges from 16° to 18° C. At the lower temperatures ammonification and nitrification go on at the same rate, but in the soils of India there tends to be an accumulation of ammonia due to the increased activities of the ammonifying bacteria at the higher temperature.

The work of Conn was continued by Brown and Smith (1912) in their investigations of the bacterial activities of frozen soils. They made quantitative determinations of the number of bacteria in the soil during the fall, winter, and spring seasons. They noted a gradual decrease in the number of bacteria as the temperature was lowered. This decrease continued with more or less variation until March 1, when there was a marked increase. They suggested that their results confirm Conn's conclusion that bacteria are alive and multiply in frozen soils. They studied what they called the ammonifying, nitrifying, denitrifying, and nitrogen-fixing powers of frozen soils. These tests were made by inoculating 100 grams of an air-dry soil with a soil infusion representing five grams of the frozen soil to be treated. The nitrogenous materials added were stirred into the unsterilized soil by means of sterile spatulas. The authors conclude that frozen soils possess a much greater ammonifying power than do non-frozen soils, and that the ammonifying power of the soil increases until the temperature almost reaches zero, when a decrease occurs, and this is followed by a gradual increase which continues until the ammonifying power reaches its maximum at the end of the frozen period.

The nitrifying power of the frozen soils was weak and the ammonifying power was strong. The nitrogen-fixing power increased with the continuance of the frozen period, being independent of moderate changes in moisture conditions. In the fall the nitrogen-fixing power of the soil increased until the soil became frozen, when it almost ceased, after which a lesser nitrogen-fixing power was established.

Brown and Smith advanced the theory, which Conn suggested in an earlier publication, that the hygroscopic water in soils, remaining uncongealed, may serve as favorable media in which the bacteria may live and multiply to a comparatively large extent.

Czermak (1912) studied the changes in the so-called physical properties of soils resulting from their subjection to low temperature, high temperature, and the action of salts. He noted that when the soil colloids were coagulated by freezing, the soil surface and the hygroscopicity were reduced, and part of the nitrogen in the soil solution was absorbed, thus reducing the amount of available nitrogen. Alternate freezing and thawing increased the coagulation of the soil colloids. The length of time was more important than the intensity of the freeze. Hoffmann (1914), however, found little or no variation in the surface area of soils due to freezing.

Lyon and Bizzell (1913) studied the effect of freezing on the nitrifying power of the soil. They noted that freezing produced a soil favorable for nitrate formation. This was attributed to the beneficial effect of low temperature in overcoming the depressing influence of the crop previously grown.

Russell and Hutchinson (1913) offer an explanation very similar to Conn's to account for the increase of bacteria in frozen soils. They suggest that the protozoa may be the hostile organisms holding the bacteria in check in the unfrozen soils.

Conn (1914) verified in later studies what he had previously found, that the increase in bacteria in frozen soils is not due to the increase in moisture content which usually occurs in winter, and that the same increase may take place in potted soils where there is no possibility of the bacteria being carried up mechanically from lower depths during the process of freezing. He suggested the possibility that the increase may not be an actual multiplication but may result from the breaking up of the masses of bacteria by freezing. He considers this to be extremely

unlikely, however, for if such was the case the count following the thawing of the ground in the spring would not be so nearly the same as it was before the freeze. He attempted to explain this increase as due to a summer and winter flora but was unable to do so. There was a surprising similarity between the predominating types of bacteria found in different soils and in the same soil at different seasons.

In an attempt to explain the presence of large numbers of bacteria in frozen soils, Harder (1916) studied the effect of heavy frosts. He found that the number of bacteria in surface soil increased markedly after heavy frosts and maintained this high average during the winter months. Contrary to the findings of Conn and of Brown and Smith, Harder noted that the increase was directly related to the moisture content. His findings verified Conn's conclusion that the bacterial flora was more or less the same thruout the year, with the exception that after heavy frosts there was an increase in the proportion of small transparent colonies. Potted soils did not show this increase even when enriched with sugars. In fact the enriched soils showed fewer organisms in the frozen than in the unfrozen samples. Harder concludes that the increase in numbers was due to mechanical transportation by moisture coming up from below during heavy frosts.

Conn (1918), in his microscopic study of bacteria and soil fungi, found that there was more or less clumping of the bacteria on the soil particles, and in certain cases the organisms occurred in large sheets one individual thick. In practically all cases there was an increase in the individual count over the group count. In one table Conn gives the results of thirty-four groups and individual counts of soils made at different periods following the addition of manure. The results show that the individual count gives an increase of 50 per cent over the group count.

The findings of Brew and Dotterrer (1917) in their study of milk were very similar to those of Conn. Brew and Dotterrer noted a higher count with the microscope than with the plate method. The difference they attributed to the clumps of bacteria that do not break up on plating.

MILK FLORA

Stiles and Pennington (1909), in their investigation with ice cream, found that the samples showed what seemed to be an increase in the bacteria count for a few days, followed by a decrease on the fourteenth

day. This was followed by a more rapid increase until the highest point was reached on the twenty-seventh day. On the thirty-fourth day the counts were about equal to those on the fourteenth day.

Hammer (1912) concludes from his studies with stored ice cream that there is no increase in the number of bacteria during storage, as judged by the number of organisms developing on agar with an incubation temperature of 37° C. He assumes, of course, that the product is kept properly hardened.

Gordon, Prescott, Heinemann, and Pease (1914) noted a marked increase in the bacteria counts of fresh ice cream over the counts of the mix as it entered the freezer.

Esten and Mason (1915) concluded from their investigations that when ice cream is kept frozen for periods of at least a month, there is no marked increase or decrease in the bacteria count as shown by litmus lactose gelatin plate counts.

Ellenberger (1919) noted an increase due to the freezing process, which he thinks may be accounted for by the breaking-up of the clumps of organisms by the beating received from the dasher during freezing. He observed no radical change in the total number of bacteria in ice cream during storage. The groups of bacteria in ice cream as determined by litmus gelatin plates and litmus milk tubes did not change noticeably during storage.

CAUSE OF DEATH OF THE CELL

Several theories have been suggested to explain the specific action which results in the death of the plant cells when exposed to low temperatures. Du Hamel and Duffon (1737) advanced the theory that death was due to the bursting of the cell wall as a result of the expansion accompanying ice formation. Senebier (cited by Göppert, 1830) also supported this view, which was later disproved by Sachs (1860) and Nägeli (1861). The formation of ice crystals within the cell and in the intercellular spaces was noted by Göppert (1830), but in no case could he find ruptured cells. He considered death a result of freezing, and as being in no way affected by the rate of thaw. Sachs concluded from his results that the degree of killing of plants at a given temperature depended on the rate of thaw.

Müller-Thurgau (1880) and Molisch (1897) were unable to detect any great difference in the amount of killing due to the difference in rate

of thaw, except in a few specific cases. They believed that death was due to the rapid withdrawal of water from the cell to form the ice crystals in the intercellular spaces during the process of freezing, and that only when freezing took place very rapidly were ice crystals formed within the cell. Unfavorable conditions appear in general to reduce the power of resistance to cold, and a deficiency or excess of water or plant food would act in the same way.

Klemm (1895) noted that the visible changes and deformations produced in the protoplasm were due to sudden changes in temperature, and were not present when the cells were subjected to gradual changes.

D'Arsonval (1901) noted that yeasts and bacteria did not lose their vital properties when placed in liquid air for weeks. He thinks the fluid in the cell is probably not solidified if the cell is not ruptured, owing to the enormous osmotic pressure in those small organisms, for if their osmotic tension is lowered by placing them in a solution of sodium chloride, potassium chloride, or glycerin, they are readily killed. He suggests the possibility of determining the osmotic pressure of any given cell by the temperature at which its vitality is destroyed.

Matruchot and Molliard (1901) subjected plants to freezing, to drying, and to the action of solutions of high osmotic concentration. They observed a marked parallelism between the action of freezing and that of drying, and they concur with Molisch in that the death of the cell is due to a rapid drying-out of the tissues.

Mez (1905) noted, in his studies of the effect of supercooling on plant tissue, that where the ice formation began at once on reaching the freezing point the killing was not so great as where there was supercooling, and the formation of ice crystals took place rapidly. He thinks that when a temperature of $-6°$ C. is reached, all solutions will crystallize out. The heat liberated by the crystallizing of the solutions and the formation of ice, will, after the cells are insulated by the ice mass, aid in keeping the temperature of the cell above that of the surrounding material. Mez holds that death is due to the direct effect of the cold.

Gorke (1907) noted that when the cell sap was frozen, certain proteids were precipitated, and that those plants that are most easily killed by freezing have their proteids precipitated at the highest temperatures. By using solutions of albumin to which had been added zinc sulfate, he was able to show that the concentration of the salts had a marked effect

on the precipitation of the proteids when the solutions were frozen. He assumed that killing from cold may be due to the precipitation of the proteids resulting from the concentration of salts in the sap as the water is removed to form the ice crystals within the intercellular spaces.

Heckel (1909) noted that anesthetics and freezing liberated coumarin very rapidly from certain plants, and that the characteristic odor was apparent from green plants in a few moments after freezing, whereas ordinarily it is not apparent until after the plants have become more or less dried. This seemed to indicate that freezing and drying affected the plant in much the same way.

In studying the relation between the density of cell saps and the freezing points of leaves, Ohlweiler (1912) noted that the extreme differences in sap density are generally accompanied by corresponding resistance to freezing. He found that when the cell structure was essentially the same, the density of the cell sap of the species would indicate its relative hardness.

Lepeschkin (1912) concludes that death in plant cells is preceded by a decomposition of the less stable protein compounds and later their coagulation. Capillary forces may play an important part in their coagulation, which in turn sets free energy that leads to the breaking down of the weaker compounds.

Maximow (1914), in his very excellent work in freezing sections of plants in solutions of various strengths of both organic and inorganic substances, found a remarkable protection to be exerted whenever the eutectic point of the substances did not lie too near the freezing point and whenever the substance was not exceedingly toxic. When the sections were immersed in these solutions and immediately frozen, as much protection was noted as when they were permitted to remain in the solution for several hours. The protective action was not in direct proportion to the osmotic pressure and the lowering of the freezing point, for it was considerably more rapid than the latter changes. Since the protective action did not depend on the time the plant was in the solution nor on the permeability of the protoplasm, Maximow concluded that the action is on the outer layer of the protoplasm, and that the withdrawal of water seems to be limited to the plasma membrane.

Chandler (1913) was able to reduce the killing temperature of plant tissue by increasing the sap density of the tissue. In the case of unripe

apples and pears, there was no indication that the rate of thawing had anything to do with the amount of killing at a given temperature.

SCOPE OF THE PRESENT INVESTIGATION

With the results of the aforesaid workers in mind, the author undertook the investigations herein reported in an attempt to answer the following questions: (1) Is there an actual growth and reproduction of the bacteria in frozen soils or solutions? (2) What is the effect of low temperature on *Bacillus radicicola* in solution, sand, and soil cultures? (3) To what is the protective action noted in solution and soil cultures due? (4) Why have so many investigators obtained such divergent results in their work with bacteria at low temperatures?

The widely varying results obtained by Conn, by Brown and Smith, and by Harder, would seem to indicate that the number of bacteria occurring in frozen soils as determined by the agar-plate method is due not to an actual multiplication of the bacteria but to some unknown condition. The findings of Harder would indicate that the moisture content is of prime importance, whereas Brown and Smith did not consider it so important. Conn found that potted soils showed the same marked increase as the field soils. Harder was unable to show this increase in the potted soils, and concluded that the bacteria were carried up from the lower depths by heavy frosts.

It is contrary to the general conception of plant and animal life to hold that bacteria will grow and multiply more rapidly at temperatures below zero than at the so-called favorable temperatures. The work of Conn, of Brown and Smith, and of Harder, gives little or no evidence to show that the bacteria do actually multiply in frozen soils, for the increase in bacteria counts noted by these investigators was based on the colonies appearing on the agar plates, and, as is well known, this method is in many cases a poor indicator of the actual number of bacteria present. The method is perhaps of value in a comparative way when comparing substances that have received more or less of the same treatment, but it seems very doubtful whether it has any real value when comparing normal and frozen soils. The results obtained by Brown and Smith are very good examples of this.

If there is not an actual increase in the number of bacteria in frozen soils — and it does not seem probable that there is — the large number of

colonies appearing on agar plates must be accounted for in some other way. As each colony on a plate may result from a single cell or from a cluster of many cells, it would seem that the increase obtained from frozen soils might be due to the breaking up of the masses of bacteria, not to an actual reproduction.

Both Conn and Harder considered this breaking-up process to be extremely unlikely, for if such was the case the count immediately after the thaw would not be so nearly the same as it was before the freeze. This objection, however, might not hold in the case of the bacteria, for they often grow in a jelly-like mass which might be ruptured by freezing but which if allowed to thaw gradually, as it would in the soil under a normal condition, might assume its original position. If such was not the case, one might expect more bacteria in recently thawed soil than in unfrozen soil. If the increase was due to a summer and a winter flora, as suggested by Conn, there should not be a rapid decrease in number of bacteria immediately after the thaw, for the soil would maintain for several days a temperature more favorable for the winter than for the summer flora. The soils that were allowed to thaw gradually in the field showed approximately the same number of bacteria as was shown by the soils before they were frozen, whereas the soils that were thawed rapidly in the laboratory showed a higher count. This would seem to indicate that the rate of thaw might be an important factor. The experiments reported herein were conducted with these considerations in mind.

METHODS OF INVESTIGATION

ORGANISMS

In the experiments dealing with the field soils, the bacterial flora of the soil was studied as it was observed on examination. A study was made of the different groups, but no marked correlation was noted between certain groups and the treatment of the soil. *Bacillus radicicola* from field pea was used for all the pure-culture work. This organism was selected because of its economical importance and habits of growth. It is easy to grow and identify, and is classed as a non-spore-bearing form. The latter character is of importance in low-temperature studies.

MEDIA

The organisms were grown in soil, sand, and solution cultures. The medium used for making the plate counts of the field soils was Conn's (1914)

asparaginate agar. The agar used in plating the *Bacillus radicicola* organism was the same as Wilson (1917) used in his studies. In the nutrient solutions the saccharose was replaced by mannite, 1 gram to a liter.

METHOD OF FREEZING, THAWING, AND PLATING THE BACTERIA

In the case of the field soils, the samples were collected in the field under sterile conditions and 20-gram portions were placed in sterile liter flasks. The samples were then taken to the laboratory in a frozen condition and treated immediately, water at the different temperatures being used to thaw the samples. Plates were poured from the 20,000 and 200,000 dilutions. Five plates were poured from each dilution in all cases. Ice and salt, liquid air, and outside temperatures, were used to freeze the laboratory samples.

The *Bacillus radicicola* organism was grown in sterile sand, soil, and water cultures. In the case of the sand and soil cultures 1-gram portions were weighed out into small, sterile, test tubes under sterile conditions, and subjected to the various treatments. The samples subjected to the liquid-air treatment were lowered into the wide-mouth Dewar bulbs containing the liquid air. Care was necessary to prevent bursting of the tube in freezing. The plates were incubated at 30° C. and counts were made at the end of four and seven days. Five plates from each of three dilutions were poured from all samples.

EFFECT OF RATE OF THAW ON BACTERIA COUNT

SOILS FROZEN IN THE FIELD

It would seem that if the large number of bacteria in frozen soils as shown by the plate method is due to the breaking up of the clusters of organisms, the rate of thaw should have some effect. In order to test this point, samples of Dunkirk clay soil containing 33 per cent of moisture were taken from the field in a frozen condition and thoroly mixed, and 20-gram portions were put into liter flasks. These samples were brought to the laboratory and plated by the dilution method. The only difference in the treatment was the temperature of the water blanks used in making the dilutions. The results are shown in table 1.

From the results shown in table 1, the rate of thaw seemed to have a marked effect as shown by the counts on agar plates. The increased count due to the sudden thawing of the soil with water at 30° C. was 200

per cent above that of the count made using water at 1° C. When the soil was allowed to thaw in the air the difference was less marked. It is interesting to note that when the temperature of the water used in plating the soil was raised to 40° C. there was always a drop in the counts. This may have been due to the death of the bacteria caused by the sudden change to higher or lower temperature. It seems more probable, however, that the higher temperatures may cause a greater or less coagulation of the masses, resulting in fewer colonies on the plates.

TABLE 1. Effect of Rate of Thaw on the Bacteria Count in Field Soils

Sample	Treatment	Bacteria per gram of dry soil
1	Water added at 1° C	3,100,000
2	Water added at 10° C	5,200,000
3	Water added at 20° C	6,000,000
4	Water added at 30° C	9,300,000
5	Water added at 40° C	6,700,000
6	Soil thawed in air at 1° C	5,000,000
7	Soil thawed in air at 10° C	6,100,000
8	Soil thawed in air at 20° C	6,300,000
9	Soil thawed in air at 30° C	6,900,000
10	Soil thawed in air at 40° C	5,800,000

SOILS FROZEN IN THE LABORATORY

Samples of soil that had been kept in a moist condition in the laboratory were used to test the effect of short periods of freezing and different rates of thaw. Counts were made of the normal soil containing 30 per cent of moisture, and the samples were then exposed to a temperature of −15° C. for twenty-four hours. In table 2 is shown the effect of short periods of freezing and different rates of thaw:

TABLE 2. Effect of Rate of Thaw on the Bacteria Count in Laboratory Soils

Sample	Treatment	Bacteria per gram of dry soil
1	Normal	4,600,000
2	Frozen 2 hours at −16° C., thawed in room	6,600,000
3	Frozen 2 hours at −16° C., water added at 30° C.	9,800,000

There was an increase of about 50 per cent in the bacteria count when the soil was thawed by adding water at 30° C., over the count from the sample allowed to thaw in the air. This would seem to indicate a breaking up of the clusters of bacteria, due to the sudden change in temperature.

EFFECT OF ALTERNATE FREEZING AND THAWING ON BACTERIA COUNT

If there is a breaking up of the masses of bacteria when subjected to extreme temperatures, then alternate freezing and thawing should have some effect. In the test to determine this point the soil was placed in two containers 10 by 6 by 4 inches in size and divided into compartments by means of strips of paper. One of the containers was brought in each day and allowed to thaw at room temperature, counts were made of the bacteria per gram of soil in one of the compartments, and the container was then placed outside to freeze again. The outer container remained frozen during the experiment. The results are shown in table 3:

TABLE 3. EFFECT OF ALTERNATE FREEZING AND THAWING ON THE BACTERIA COUNT

Day	Treatment	Bacteria per gram of dry soil
First	Normal	4,500,000
Second	Frozen and thawed	7,000,000
Third	Frozen and thawed	8,100,000
Fourth	Frozen and thawed	10,200,000
Fifth	Frozen and thawed	11,200,000
Sixth	Frozen and thawed	11,100,000
Seventh	Frozen and thawed	11,900,000
Eighth	Continually frozen	8,900,000

The results shown in table 3 would seem to indicate that alternate freezing and thawing did have some effect, until the fifth day at least. After that there was little or no increase. There is one thing that must be taken into consideration in all these results, and that is the possibility that some of the bacteria may be killed by freezing and that the number as shown by the plate count may represent a greater breaking-up of the masses than the results would otherwise indicate. Inasmuch as the pots were only 4 inches deep, and the samples from which the counts were made

represented the bottom as well as the top of the soil, the increase could not have been due to the drawing-up of the organisms from the lower depths, as suggested by Harder.

EFFECT OF TIME, TEMPERATURE, AND RATE OF THAW ON BACTERIA COUNT

IN CLAY SOILS

The effect of the time of freezing and the rate of thaw on the number of bacteria in frozen soil, and on the bacteria in the dilutions from the soil, is shown in table 4. In the test to determine the latter, samples were taken from the flasks containing the last dilution — the one that was plated out — and were frozen for different lengths of time.

TABLE 4. Effect of Time, Temperature, and Rate of Thaw on the Bacteria Count in Clay Soils

Sample	Treatment	Bacteria per gram of dry soil
1	Normal soil, water blank at 30° C	7,500,000
2	Normal soil, water blank at 0° C	6,800,000
3	Frozen 2 hours at −15° C., water added at 0° C.	5,600,000
4	Frozen 2 hours at −15° C., water added at 15° C.	7,100,000
5	Frozen 2 hours at −15° C., water added at 25° C.	8,200,000
6	Frozen 2 hours at −15° C., water added at 40° C.	5,800,000
7	Dilution of sample 1, frozen 1 hour	6,400,000
8	Dilution of sample 1, frozen 12 hours	4,300,000
9	Dilution of sample 1, stood 12 hours	5,700,000
10	Frozen in liquid air at −190° C. 1 minute	8,000,000
11	Frozen in liquid air at −190° C. 30 minutes	7,100,000
12	Frozen in liquid air at −190° C. 6 hours	3,400,000

The temperature of the water used in making the dilution seemed to have a marked effect on the bacteria in the frozen soil and a slight effect on the bacteria in the unfrozen soil. These results seem to indicate that the present method of making plate counts should be standardized, and that the temperature of the water blanks used is one of the factors that must be controlled if the results are to be comparable.

When the soil was frozen in liquid air at a temperature of −190° C. for one minute, there was a noticeable increase in the bacteria count which could not be accounted for by actual growth and multiplication.

When the exposure to liquid air was continued for several hours there was a decrease in the number of bacteria.

The effect of freezing seemed to be less harmful on the bacteria in the soil than on those in the soil dilution in the water blanks. When the freezing was continued for 12 hours there was a marked decrease in number of bacteria. It would seem that the more concentrated soil solution acted as a protective agency, and when the solution was weakened the effect of low temperatures was more marked.

IN SANDY SOILS

The soil used by Harder in his work was a rich sandy loam, and as his results were so marked it was thought advisable to compare a sandy loam soil with the Dunkirk clay soil. The results obtained when a light sandy soil was used are shown in table 5:

TABLE 5. EFFECT OF TIME, TEMPERATURE, AND RATE OF THAW ON BACTERIA COUNT IN SANDY SOIL

Sample	Treatment	Bacteria per gram of dry soil
1	Normal soil, water blank at 24° C.	6,400,000
2	Frozen in liquid air 5 minutes, thawed at 1° C.	4,800,000
3	Frozen in liquid air 5 minutes, thawed at 10° C.	5,400,000
4	Frozen in liquid air 5 minutes, thawed at 24° C.	6,800,000
5	Frozen in liquid air 5 minutes, thawed at 30° C.	5,600,000
6	Frozen in liquid air 5 minutes, thawed at 38° C.	4,800,000
7	Frozen in liquid air 1 minute, thawed at 24° C.	5,400,000
8	Frozen in ice and salt 1 minute, thawed at 24° C.	7,200,000
9	Frozen in ice and salt 2 hours, thawed at 24° C.	14,000,000
10	Frozen in liquid air 2 hours, thawed at 24° C.	11,600,000

There was the same increase in the bacteria count due to the rate of thaw as noted in the earlier work, and a drop in the bacteria count when the temperature of the water blanks was raised to 30° C. and above. When the soil was frozen in ice and salt for one minute, there was an increase of nearly one million bacteria per gram of soil over the count for the normal soil. When the soil was frozen for two hours in ice and salt, the count increased to 14,000,000, showing an increase of 120 per cent over the count for the normal soil. When the soil was frozen in liquid air for two hours there was also a marked increase. It is evident that this

increase could not have been due to growth and reproduction of the bacteria in the unfrozen soil solution, as suggested by Brown, but that it must have been due to the breaking up of the clumps of bacteria.

The results from the liquid-air treatment would indicate that there is a breaking up of the clusters of bacteria sufficient to show a marked increase above the number in normal soil. When it is considered that probably many of the bacteria were killed by the low temperature, it seems evident that the breaking-up process must be even greater than the plate counts indicate.

AMMONIFICATION AND NITRIFICATION IN FROZEN SOILS

In order to test the bacterial activities in frozen soils more carefully, the ammonifying and nitrifying powers of frozen soils were studied. Peptone and casein were added to the soils at the rate of ½ gram to 100 grams of soil. The determinations of ammonia were made at the beginning of the experiment and at the end of five days. One-half of the samples were placed in the incubator at 22° C., and the other half were kept frozen during the experiment. The determinations of nitrates were made at the beginning of the experiment and at the end of four weeks. The samples were treated the same as in the ammonification experiment. The results are shown in table 6:

TABLE 6. Ammonification and Nitrification in Frozen and in Unfrozen Soils

Treatment	Parts per million of dry soil			
	Ammonia nitrogen		Nitrate nitrogen	
	At beginning of experiment	At the end of 5 days	At beginning of experiment	At the end of 4 weeks
Soil and peptone, frozen	65.4	68.7	112	118
Soil and peptone, incubated	65.4	382.9	112	253
Soil and casein, frozen	56.1	55.3	112	116
Soil and casein, incubated	56.1	336.9	112	237

These results show that there is little or no change going on in the soil solution when it is in a frozen state. This indicates that no protoplasmic activity, such as growth or reproduction, is taking place.

EFFECT OF LOW TEMPERATURE ON BACILLUS RADICICOLA

Since little or no work has been done in studying the effect of low temperature on pure cultures of the soil bacteria, it was deemed advisable to use the non-spore-bearing organism *Bacillus radicicola*, because of its economic importance. Since this is the organism used for inoculating the legume plants, it was thought that if low temperature does have a marked effect on its development, this fact might throw some light on the soil-inoculation question.

The method employed was to grow the bacteria in pure cultures in nutrient solutions, sand, and soil, and then subject them to different degrees of temperature for varying lengths of time. The nutrient solution used was the one recommended by Wilson (1917) in his studies of *Bacillus radicicola*. The saccharose in the original solution was replaced by mannite, using 1 gram per liter. The larger amount of sugar tended to increase the viscosity of the growth, which rendered it less favorable for quantitative work. The soil used was of the Dunkirk series. Counts were made by the agar-plate method. The medium used was the same as the nutrient solution mentioned above, with the addition of 1.5 per cent agar. The 10 grams of soil — or, in the case of the nutrient solution, 1 cubic centimeter — was added to water blanks until the desired dilution was obtained. Five plates were poured from each dilution, and three dilutions were used in all cases for pouring the plates. Separate counts were made in all cases and the averages of the five plates were taken for the final counts. The results in table 7 represent the findings on 1350 plates.

All freezing was done in thin-walled glass tubes. In the solution tests the bacteria were grown in the test tubes in which they were later frozen. Five-hundred-gram portions of the sterile soil and sand were inoculated with the bacteria and maintained for one week at 25° C. Tests were made of these samples by weighing out one-gram portions into sterile tubes and subjecting them to the low temperatures. The samples were then added to 500-cubic-centimeter water blanks, in a frozen condition. An attempt was made to keep uniform all conditions that were not being studied. By lowering the tubes gradually into the liquid air it was possible to freeze them without breaking the glass.

In interpreting the results shown in table 7, the fact must be kept in mind that the present method of determining the number of bacteria is very

unsatisfactory, especially when working with frozen soils. The colonies on the plate may represent a single cell or a mass of cells, and, altho the number of colonies may be greater after freezing, the investigator is not at all sure that a great many of the organisms have not been killed and that the seeming increase is due to the breaking up of the clusters.

TABLE 7. Influence of Low Temperature on Bacillus radicicola in Solution, Sand, and Soil Cultures

Sample	Treatment	Bacteria per cc. of solution	Bacteria per gram	
			Sand	Soil
1	Normal soil	3,000,000	3,700,000	400,000,000
2	Frozen with ice and salt at −15° C. 3 minutes	1,400,000	5,200,000	470,000,000
3	Frozen with ice and salt at −15° C. 1 hour	1,000,000	3,200,000	500,000,000
4	Frozen with ice and salt at −15° C. 2½ hours	750,000	3,000,000	520,000,000
5	Frozen with ice and salt at −15° C. 6 hours	600,000	2,800,000	400,000,000
6	Frozen in liquid air at −190° C. instantaneously	1,000,000	2,700,000	470,000,000
7	Frozen in liquid air at −190° C. 30 minutes	750,000	2,200,000	380,000,000
8	Frozen in liquid air at −190° C. 1 hour	620,000	2,100,000	360,000,000
9	Frozen in liquid air at −190° C. 2½ hours	600,000	2,600,000	530,000,000
10	Frozen in liquid air at −190° C. 6 hours	450,000	2,100,000	400,000,000

In table 7 it is shown that there is a gradual decrease in the number of bacteria in the solution as the degree of cold and the time of freezing is continued. In the sand cultures there was a marked increase in the number of bacteria when frozen for a few minutes at −15° C. This would indicate a breaking up of the clusters of bacteria that may surround the sand particles. A slight protective action was present in the sand and soil cultures that was not noticed in the solution cultures. The length of time of freezing had very little effect when liquid air was used. In sand the total count was slightly below normal, and remained so during the test.

The results obtained with the soil cultures are interesting and give some idea of what may be expected in such work dealing with the bacterial

flora of the soil. The results show no difference between the total counts of the normal soil and those of the samples that had been exposed to the temperature of liquid air for six hours, or those frozen with ice and salt for six hours. The samples exposed to the temperatures of −15° and −190° C. both showed a marked increase over that of the normal soil, again indicating a breaking-up process. It would seem that the non-spore-bearing bacteria, such as *Bacillus radicicola*, are able to withstand the effect of low temperature of the soil. Careful work on the bacterial-activity problem would doubtless verify this supposition. All evidence points to the probability that the effect of freezing on the soil is physical, not bacteriological.

INFLUENCE OF CONCENTRATION OF THE MEDIUM ON THE DEATH OF BACTERIA AT LOW TEMPERATURES

The results obtained with *Bacillus radicicola* in solution and soil cultures would seem to indicate that the concentration of the solution surrounding the bacteria had something to do with the ability of the bacteria to withstand low temperatures. The work of Maximow (1914) showed that such a protective action was evident when plants were placed in solutions of high concentrations. This protective action noted in the soil may have been due to the concentration of the soil solution or to some other factor such as the influence of surface tension on the large and the small soil particles.

If the death of the bacterial cell when frozen is due to the withdrawal of water from the semi-permeable membrane, or outer layer of the cell, resulting in the precipitation of the proteids, then it should be possible to overcome this harmful effect of freezing by increasing the concentration of the cell sap. The greater the concentration of the cell sap within the cell, the greater would be the osmotic pressure, the greater the power of inhibition, and the greater the resistance of the cell to low temperatures.

There is a possibility that the surface tension may be so great on the small particles that it would prevent the freezing of the solution. But when one considers that a pressure of 2600 atmospheres is necessary to prevent starch grains from absorbing water, and a pressure of 13,000 atmospheres is necessary to prevent water from freezing at −20° C., one realizes that if the surface tension does play an important part in the soil solution the pressure must be very great.

INFLUENCE OF GLYCERIN

If the protective action is due to the greater concentration of the solution, the addition of sugar or similar substances should have the same effect. In order to test the influence of the concentration of the medium, a very weak nutrient solution was prepared as follows: Dibasic potassium phosphate 0.05 gram, magnesium sulfate 0.05 gram, sodium chloride 0.05 gram, calcium sulfate 0.05 gram, distilled water 1000 cubic centimeters. Glycerin was added at the following rates: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 15 per cent.

Test tubes containing 10 cubic centimeters of the above nutrient solution with glycerin added in increasing amounts, were inoculated with a culture of *Bacillus radicicola* and incubated for five days at 25° C. At the end of that period, counts were made of the number of bacteria per cubic centimeter in each tube, by means of the agar plates. The tubes were then frozen for thirty minutes in ice and salt at a temperature of −15° C. At the end of the half hour the tubes were thawed by placing them in water at 15° C., and were plated as in the preceding experiments. Care was taken to have all conditions uniform, for the earlier findings indicate that a difference in the temperature of the water blanks or in the rate of thaw might make a difference in these results. All counts were made at the end of five days incubation. Nine plates were poured from each tube and the experiment was run in triplicate, so that each count represents the average of twenty-seven plates.

TABLE 8. INFLUENCE OF GLYCERIN ON THE DEATH OF BACTERIA WHEN FROZEN AT −15° C.

Sample	Glycerin (per cent)	Normal bacteria count	Bacteria count after being frozen for 15 minutes	Per cent of bacteria killed
1	None	5,600,000	236,000	96
2	0.01	12,300,000	984,000	92
3	0.05	13,600,000	1,768,000	87
4	0.1	17,000,000	10,000,000	41
5	0.5	21,000,000	11,550,000	45
6	1.0	23,000,000	23,100,000	0
7	5.0	24,300,000	28,200,000	0
8	10.0	520,000	546,000	0
9	15.0	No growth	No growth	.....

The results obtained when glycerin was used in varying amounts are shown in table 8. A remarkable protective action was exerted by the glycerin when the concentration was 1 per cent and above. With decreasing amounts of glycerin down to 0.01 per cent, there was a rapid increase in the percentage of bacteria killed. When the concentration of glycerin was raised to 5 and 10 per cent, there was an actual increase in the number of bacteria, as is shown by the colonies on the agar plates. This seeming increase was perhaps due to the breaking up of the clumps of bacteria. It is possible that in the nutrient solution containing 1 per cent of glycerin a great many of the organisms were killed, and that the high counts were due to the breaking up of the masses of bacteria. There was little or no growth in the nutrient solutions containing 15 per cent of glycerin.

INFLUENCE OF DEXTROSE

The results from the higher concentrations of media using glycerin and dextrose verified the finding of Maximow in his work with red cabbage and *Tradescantia discolor*. So long as the temperature of the frozen medium was maintained above the eutectic point of the substance used, the protective action was noted. If the above findings hold true in regard to the bacterial cell, the concentration of the medium should have no effect when the temperature is lowered to a point at which the glycerin and the dextrose will crystallize out. To test this latter point, the bacteria in the solutions described above were subjected to a temperature of −190° C., using ice and salt.

The results obtained with the different concentrations of media when exposed to a temperature of −190° C. using liquid air, and to a temperature of −15° C. using ice and salt, are given in table 9. There was the same marked protective action due to the increased concentration of the medium when the solutions were frozen in ice and salt for two hours, as was shown by glycerin (table 8). When the bacteria were subjected to the temperature of liquid air for five minutes the concentration of the medium had little or no effect. These results would seem to substantiate the foregoing theory, that when the temperature is lowered below that of the eutectic point of the sugars added, the concentration of the medium has no effect.

In table 9 the percentage of bacteria killed is based on the number of colonies growing on the plates poured before and after treatment of the

samples. The normal is considered as 100 per cent and the decrease is given as per cent killed. It is interesting to note that the concentration of the medium had no effect when the temperature was lowered below the eutectic point of the sugar, but at temperatures about the eutectic point the sugar showed a marked protective action.

TABLE 9. Influence of Dextrose on the Resistance of Bacteria to Low Temperatures

Sample	Dextrose (per cent)	Normal bacteria count	Bacteria count when frozen in liquid air for 5 minutes	Per cent of bacteria killed	Bacteria count when frozen in ice and salt for 2 hours	Per cent of bacteria killed
1	None	No growth	.....	.....	.....	.....
2	0.01	880,000	570,000	35	17,600	98
3	0.05	1,960,000	1,240,000	37	98,000	95
4	0.1	3,600,000	2,400,000	33	398,000	89
5	0.5	4,700,000	2,490,000	47	1,220,000	74
6	1.0	16,400,000	9,480,000	42	6,890,000	58
7	5.0	24,100,000	16,390,000	32	15,660,000	35
8	10.0	11,300,000	8,140,000	28	10,850,000	4
9	15.0	8,200,000	4,670,000	43	8,000,000	2

GENERAL DISCUSSION

It is evident from the data given that the so-called increase in the number of bacteria in frozen soils is due, not to an actual multiplication and growth of the bacteria in the soil solution as suggested by Conn, but rather to a breaking up of the clumps of bacteria which results in an increased number of colonies on the agar plates. This view is substantiated by the fact that the same increase and decrease in counts may be obtained in the laboratory by freezing the soil for a few minutes and then thawing it out by means of water blanks at different temperatures.

The fact that the same marked increase may take place in soils when frozen in liquid air at a temperature of −190° C. for a few hours, invalidates Brown's theory that the increased growth takes place in the uncongealed hygroscopic water of the soil, for at this low temperature the amount of water remaining uncongealed could hardly be considered sufficient for the favorable growth of bacteria.

It is interesting to note that Conn's counts for the frozen soil were higher than those for the unfrozen soil, whereas the counts of Brown and Smith for the frozen soil were in all cases except one far below those for the unfrozen soil, and in the sample taken on March 1, in which the count was above normal, the moisture content had been increased from 15.76 to 26.57 per cent. Regardless of the fact that three samples out of the four taken showed a lower bacteria count than the normal soil, Brown and Smith concluded that their results substantiated the conclusion of Conn that bacteria do reproduce in increasing numbers in frozen soil.

It is interesting to note the very close correlation that can be drawn between the increase in the number of bacteria in frozen soils and the increase in the individual over the group counts, as shown by Conn in recent publications (1917 and 1918). In both cases the increase was between 25 and 50 per cent, the individual count showing the greater gains. The gains obtained by the author with the frozen samples are in very close keeping with the preceding.

The moisture content and the rate of thaw are the important factors that determine to a large extent the breaking-up process. This point is well brought out in the work of Harder. When the frozen soil was thawed rapidly by rain there were a large number of organisms occurring in the thawed soil, but when the thaw was gradual the number dropped back to about normal. The only high count obtained by Brown and Smith can be explained in somewhat the same manner. Their sample taken on February 11 showed a moisture content of 15.7 per cent and a bacteria count of 4,744,000 colonies; whereas their sample taken on March 1 showed a moisture content of 26.5 per cent and a bacteria count of 16,870,000. Between these two dates the moisture content rose 68 per cent, which would indicate that there had been a thaw followed by a freeze, resulting in a breaking up of the masses of bacteria.

That frozen soil is not as favorable a medium as unfrozen soil for the growth of bacteria is shown by the results obtained by Harder when soils were treated with dextrose. His results show a retardation in growth caused by low temperature, which even a much higher moisture content was not sufficient to counteract. They substantiate the theory that the increased count in frozen soils is due to a breaking up of the clumps of bacteria, for such an increase should be more marked in soils having a high moisture content and this condition was found to be true

in almost all cases. When the soil was thawed rapidly by rain, the count in the thawed soil was very much higher than when the thaw was gradual, indicating a breaking-up process due to the rapid thaw. When the conditions for an actual increase and growth were brought about by the addition of dextrose to the soil, the unfrozen sample showed a much greater increase in bacteria content than did the frozen sample. All this would seem to prove that frozen soil is not as favorable a medium as unfrozen soil for the growth of bacteria. That the increase is not due to mechanical transportation by moisture coming up from below during heavy frosts was shown by Conn in his work with potted soils, and also in the results herein reported, in which the same marked increase was obtained when small amounts of the soil were frozen in test tubes and an entire portion was used in making the dilutions.

The conclusions of Brown and Smith in regard to bacterial activities in frozen soils have not sufficient evidence to give them weight. It is interesting to note how their idea of a summer and a winter flora has been made to fit in with their results. Altho they concluded that the ammonifying power of the frozen soil is increased, the smallest amount of ammonia was produced from the sample taken during the coldest period of the tests. When it is considered that Brown and Smith used a soil infusion representing 5 grams of the frozen soil to inoculate 100 grams of air-dry soil, the results are not surprising. It would seem that the experiment under such conditions could better be called a study of the effect of storing air-dry soil on the bacterial flora therein, inasmuch as the number of bacteria present in the air-dry soil was several times that in the 5 grams of frozen soil. Duplicate tests might perhaps have eliminated the marked variations obtained by Brown and Smith. The tests that should have shown the difference, if there really was one, were those on nitrification, for the condition surrounding those tests are more easily controlled, and the measured product, nitrates, is not so easily lost as are some of the other products. The nitrification results of Brown and Smith indicated that if there was a difference their method was not sufficiently accurate to show it. It would seem that a similar conclusion may be applied to all their results.

Brown and Smith concluded from their nitrification results that the nitrifying power of the soil was rather weak. This is contrary to the findings of Lyon and Bizzell, who noted a beneficial effect due to freezing.

If there was active growth and reproduction of the ammonifying and other types of organisms in the soil, one would expect a protoplasmic activity that would be indicated quantitatively or qualitatively by the formation of ammonia and similar products. As no change was noticeable in the frozen soil, even when large amounts of nitrogenous materials were added, it would seem as if bacterial activities had ceased.

If the bacterial flora of the soil can withstand the low temperature of liquid air for several hours, it seems probable that the mild temperatures to which our soils are subjected during the winter would have little or no effect in changing the bacterial flora therein, and it will require more careful work than has yet been done on the bacterial activities in frozen soils to prove that such a change does take place.

The results obtained by investigators in the field of soil colloids seem to indicate a physical change in the soil due to freezing. It is probably this physical change in the soil and its colloids that influences the bacterial activities, rather than a change in the bacterial flora of the soil.

There is a possibility that pressure may play some part in cell division, for the soil is subjected to considerable pressure during the formation of ice from the soil solution. Kny (1896) noted that pressure actually induced cell division in the pith of Impatiens, and that very pronounced pressure will cause the periclinal divisions of the cambium to cease and anticlinal ones to appear. In many cases he found that pressure induced he formation of cell walls at right angles to its line of action. The close correlation between the high moisture content and the large number of bacteria occurring in the frozen soil seems to favor this theory, for the greater the moisture content, the greater would be the pressure. The fact that small quantities of soil placed loosely in test tubes and frozen showed the same increase, would, however, seem to indicate that the increase was not due to pressure.

The wide variations found by many careful investigators in their studies of the influence of low temperatures on bacteria, may be accounted for by the difference in the concentration of the media in which the bacteria were grown. The fact that 98 per cent of the organisms growing in a very rich nutrient solution survived the low temperature, would seem to support the theory that the higher the concentration of the medium surrounding the cell, the higher will be the concentration of the

cell sap within the cell, which in turn enables the cell to resist freezing at temperatures sufficiently low to freeze the cells containing a weaker solution.

SUMMARY

The increase in the bacteria counts of frozen soils, as determined by the agar-plate method, is due to the breaking up of the clumps of bacteria, not to growth and multiplication.

There seems to be no change in the bacterial flora of the soil due to freezing. The bacterial activities are influenced only in so far as the physical properties of the soil are affected.

The concentration of the medium, the length of time of the exposure, and the degree of cold, are the three important factors that determine the power of resistance of the bacteria to low temperature.

The protective action due to the concentration of the medium seems to be effective only in cases in which the eutectic point of the substances in solution is below the temperature of the exposure. When the bacteria were exposed to the temperature of liquid air the concentration of the medium had less effect.

The death of the bacterial cell when exposed to low temperature seems to be due to the withdrawal of water from the semi-permeable membrane, or outer layer of the cell.

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